TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 1029/00196

U S APPLICATION NO (If known, see 37 CFR 1 5): 09/529217

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP98/06286	2 October 1998	8 October 1997

TITLE OF INVENTION

MEANS FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF MICROBIAL POPULATIONS POTENTIALLY PRESENT IN A SAMPLE

APPLICANT(S) FOR DO/EO/US

Guillot, Emmanuelle , Urbain, Vincent , Manem, Jacques , Rittmann, Bruce E., Stahl, David A., Flax, Jodi , Wagner, Michaël

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. x This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. § 371.
3. x This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. x A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5 x A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. x has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. x A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C 371(c)(4)).

10. ☐ A translation of the Annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. x An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. x A FIRST preliminary amendment.
13. ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter

16. x Other items or information: Preliminary search report, International Search Report, International Preliminary Examination Report

U S APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/529217	PCT/EP98/06286	1029/00196

x The following fees are submitted:

	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO.....**$840.00** International preliminary examination fee paid to USPTO (37 CFR 1.482) ....**$670.00** No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))..... **$760.00** Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... **$970.00** International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... **$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$840	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	23- 20 =	3	X **$18.00**	$ 54	
Independent Claims	1- 3 =	0	X **$78.00**	$	
Multiple dependent claim(s)(if applicable)			+ **$260.00**	$	
TOTAL OF ABOVE CALCULATIONS =				**$894**	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28)				$	
SUBTOTAL =				**$894**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
TOTAL NATIONAL FEE =				**$894**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				**$894**	
				Amount to be: refunded	$
				charged	$

a. x A check in the amount of $894 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. **22-0185** in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. x The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **22-0185**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status

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SIGNATURE

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37,134
REGISTRATION NUMBER

09/529217

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	:	
	:	
Emmanuelle Guillot et al.	:	
	:	
Serial No.: To be assigned	:	Art Unit: To be assigned
	:	
Filed: Herewith	:	Examiner: To be assigned
	:	
For: MEANS FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF MICROBIAL POPULATIONS POTENTIALLY PRESENT IN A SAMPLE	:	Atty Docket: 1029/00196

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to initial examination, please amend the above-captioned case as follows.

IN THE CLAIMS

Please amend the claims as follows.

Claim 3, line 1, delete "or 2".

Claim 5, line 1, delete "any one of the preceding claims" and insert ---Claim 1---.

Claim 6, line 1, delete "any one of the preceding Claims" and insert ---Claim 1---.

Claim 7, line 1, delete "any one of the preceding claims" and insert ---Claim 1---.

Claim 9, line 1, delete "or 8".

Claim 11, line 1, delete "any one of the preceding claims" and insert ---Claim 1---.

Claim 12, line 1, delete "any one of the preceding Claims" and insert

---Claim 1---.

Claim 13, line 1, delete "any one of the preceding claims" and insert ---Claim 1---.

Claim 14, line 1, delete "any one of the preceding claims" and insert ---Claim 1---.

Claim 16, line 1, delete "any one of the preceding claims" and insert ---Claim 1---.

Claim 17, line 1, delete "any one of the preceding claims" and insert ---Claim 1---.

Claim 18, line 1, delete "any one of the preceding claims" and insert ---Claim 1---.

Claim 19, line 1, delete "any one of the preceding claims" and insert ---Claim 1---.

Claim 20, line 1, delete "any one of the preceding claims" and insert ---Claim 1---.

Claim 21, line 1, delete "any one of the preceding claims" and insert ---Claim 1---.

Claim 22, line 1, delete "any of the preceding claims" and insert ---Claim 1---.

Claim 23, line 1, delete "any of the preceding claims" and insert ---Claim 1---.

REMARKS

The claims have been amended to eliminate multiple dependency and to improve their format. None of these amendments is believed to involve any new matter. Accordingly, it is respectfully requested that the foregoing amendments be entered, that

the application as so amended receive an examination on the merits, and that the claims as now presented receive an early allowance.

Respectfully submitted,

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Date: 4-10-00

TITLE:

Means for qualitative and quantitative analysis of microbial populations potentially present in a sample

This invention may be generally described as a means of qualitative and quantitative analysis of microbial populations potentially present in a sample. More specifically, it relates to a means of qualitative and quantitative analysis using RNA-targeted oligonucleotide probes.

The analysis of microbial populations potentially present is required for many types of solid and fluid samples. Some notable examples are those samples obtained from a natural or biological environment such as natural water or hot springs; samples taken from humans or animals such as blood, urine, vaginal and intestinal flora; and samples from urban, agricultural and industrial environments such as food products, industrial water, industrial effluents, municipal wastewater, industrial sludge, fermentation media, aerosols, filters or air from air conditioning systems.

Various laboratory techniques have been developed for the qualitative and quantitative analysis of microbial populations potentially present in a given sample.

One familiar technique involves a count of the microorganisms that develop after the sample (or an extract thereof) is cultured on various selective nutrient media under standard conditions. This technique is simple but entails significant risks of errors and artifacts (low specificity of morphological criteria, inability to detect viable but non-culturable microorganisms, inability to detect slow-growing microorganisms, need to maintain viability of bacteria between collection and enumeration). Moreover, this technique generally requires longer than 24 hours to yield results.

A second technique, which entails the measurement of the activity of one or more enzymes, allows a rapid quantification of populations of living microorganisms (culturable microorganisms and/or microorganisms in a viable but non-culturable form). This technique can be used, in particular, to monitor a set of populations, but does not achieve very high levels of specificity or sensitivity.

A third technique using immunological probes often requires a growth step and thus requires longer than 24 hours to yield results. Moreover, it frequently lacks both sensitivity and specificity (misidentification may occur due to cross-reactions).

The most recent techniques are based on the use of specific DNA probes, which are generally labeled to permit detection after hybridization with their targets. Two main categories of oligonucleotide probes have been developed: those that target DNA and those that target RNA (ribosomal RNA or messager RNA).

DNA-targeted probes, although potentially highly specific, have the drawback of low sensitivity due to the few copies of the target DNA genes in each microbial cell. Although the use of PCR (polymerase chain reaction) to amplify the target DNA sequences before detection can compensate for the lack of sensitivity of the DNA probes, it has several drawbacks of its own: for example, the presence of inhibitors can lead to false-negative reactions, while carry-over or similar contamination can lead to false-positive reactions. In contrast, the use of RNA-targeted probes prevents from such drawbacks. In particular, because of the large number of copies of *r*RNA that occur naturally in a microorganism (actively growing cells may contain 10^4 ribosomes, each a potential probe target), the use of *r*RNA-targeted probes does not require the amplification step, thereby overcoming the constraints and artifacts associated therewith. The advantage of targeting *r*RNA is that about 85-90 percent of the total RNA in a typical cell is *r*RNA.

The hybridization of RNA-targeted probes can be achieved either after cell lysis, extraction and purification of the total nucleic acids of the

sample, or *in situ* on whole cells, generally after fixation (permeabilization) of the membrane (or wall) of the microorganisms potentially present in the sample.

However, cell lysis and the ensuing extraction and purification of the nucleic acids particularly total RNA, are delicate and time-consuming manipulations that require costly apparatus, trained personnel and strict experimental conditions, notably the prevention of contamination by nucleases during the procedure. This technique further implies the use of a solid support, such as a nylon membrane, onto which the purified nucleic acids are immobilized in such a way one can discriminate between them (*e.g.* dot-blot, slot-blot). It most generally also implies the use of radioactive probe labels, the handling of which requires special care. The cell lysis technique for RNA hybridididization is therefore ill-suited to use in routine analysis either in industry or in biological laboratories.

In situ hybridization in whole cells overcomes the need for preliminary extraction of the target nucleic acids by cellular lysis with all its associated disadvantages. The FISH (Fluorescent In Situ Hybridization) process, which employs fluorescence-labeled *r*RNA probes, is one existing *in situ* technique. This type of technique, generally involving fluorescence microscopy, provides a fast and sensitive qualitative analysis on many types of sample. Today, *r*RNA-targeted probes thus hybridized *in situ* with their target within whole cells can be quantified directly on the sample (flow cytometry, microscopy), although the method is not entirely satisfactory: quantification directly on the sample is technically costly, time-consuming, requires trained personnel and does not permit an accurate quantification of hybridized probes when the sample is complex and non-uniform (*e.g.* floc or aggregates formed by filamentous bacteria in sewage treatment sludge; samples containing naturally fluorescent microorganisms). As a result, the technique of *in situ* hybridization in whole cells using fluorescence-labeled oligonucleotide probes has, to date, remained an essentially qualitative technique that does not provide reliable quantitative results.

To meet the need for industrial-caliber performance on samples that can be complex and/or non-uniform, this invention provides a means for analyzing, both qualitatively and quantitatively, the microbial populations potentially present in a biological sample, said means overcoming the disadvantages of prior art techniques.

The object of this invention is, therefore, a method of qualitative and quantitative analysis of the microbial population(s) potentially present in a sample, characterized in that it comprises:

- contacting the microorganisms potentially present in said sample with at least one RNA-targeted oligonucleotide probe, hereafter called specific probe, able to target a desired microbiological population, under conditions favourable to *in situ* hybridization in whole cells,

- extracting, by separation from their target and elution outside said cells, those probes which have become hybridized,

- detecting the extracted probes and measuring the amount thereof or their respective amounts.

The present invention thus advantageously enables the extraction of said probes without destruction of said cells.

As used herein, the term "microbiological population" (or "microbiological domain") means the set of microorganisms that a given probe is able to recognize by recognition of an RNA target sequence present in each member of said set. The approach is based on oligonucleotide hybridization probes complementary to RNA sequences that are diagnostic for selected phylogenetic groups which correspond, to varying degrees, to a target region typical of a type of microorganism or a whole group of microorganisms. Any probe enabling said contacting step is appropriate for the implementation of the method according to the invention. The choice of the specific probe(s) is directly related to the analysis desired for said sample. Probes can *e.g.* be composed of oligonucleotide sequences that can distinguish between the primary kingdoms (eukaryotes, eubacteria, archaebacteria) and between closely related organisms (the group of

Ammonia-oxidizing β-Proteobacteria, the genus *Nitrobacter* or Acinetobacter or the species *Fibrobacter intestinalis,* the species *Escherichia Coli*). Probes with finer phylogenetic resolution can be derived by using the existing collections of RNA sequences. Many examples of such RNA-targeted probes are described in the prior art such as patents or patent applications, scientific publications *e.g.* Los Reyes *et al.* 1997, Appli. Environ. Microbiol. Vol. 63 n°3 p.1107-1117 ; Mobarry *et al.* 1996, Appli. Environ. Microbiol. Vol.62 n°6 p.2156-2162 ; Wagner *et al.* 1994, Appli. Environ. Microbiol. Vol. 60 n°3 p.792-800 ; Kane *et al.* Appli. Environ. Microbiol. Vol. 59 n°3 p. 682-686. Other examples of such probes can also be designed by the person skilled in the art. Advantageous probes are those which target ribosomal RNA (rRNA). Examples of such advantageous probes include Nb1000 (SEQ ID N°1) and Nso 1225 (SEQ ID N°2).

The method of the invention gives particularly accurate quantitative results when the cell numbers in said sample are equal to or greater than approximately 10^3 or 10^4 cells per ml. If desired, the microorganism concentration of a liquid sample can be increased by filtration or any other technique prior to implementing the method of the invention.

In a preferred arrangement of the invention, said microorganisms potentially present in the sample are also contacted with at least one probe, hereafter called "universal probe", serving to normalize the results obtained with probes targeting specific phylogenetic groups of microorganisms ("specific probes"). The amount of a specific probe in said sample may then be expressed as a ratio of the amount of said universal probe. Such an universal probe may thus enable the expression of *e.g.* the specific target *r*RNA as a percentage of the total *r*RNA. Examples of such "universal probes" include probes specific for any microorganism, or probes specific for bacteria, or for eukaryotes. Such "universal probes" are well-known in the art and any of them can be used as long as it enables said contacting step, and allows the desired "specific probe" normalization. Such a "universal probe" is

used in the method according to the invention similarly as a "specific probe", and accordingly is advantageously a *r*RNA-targeted probe.

It may be advantageous to extract the microorganisms potentially present in said sample therefrom, in particular by centrifugation, prior to proceeding with any step of the method of the invention. One reason to proceed in this manner is to remove the background noise that a sample of complex composition can generate. Another reason may be to place into solution the microorganisms potentially present in a solid, gazeous or viscous sample.

According to one embodiment of the invention, said contacting step is performed after the cells are made to undergo a fixation step (or permeabilization step) essential for maintaining their morphological integrity, and which makes the microorganisms potentially present in said sample permeable to short oligonucleotide probes (*ca* 15-25 nucleotides). This fixation step allows the probes to penetrate inside the microbial cells without affecting the integrity thereof, thereby attaining their target or targets *in situ*. Where applicable, said sample is homogenized prior to said fixation step in order for said at least one probe to have access to all microbial populations potentially present in the sample.

Said fixation is advantageously achieved by incubating said cells in a paraformaldehyde solution that is less than 10%, preferably around 4%, for 3 to 12 hours at 4°C. This fixation procedure is more particularly adapted to Gram-negative bacterias. For certain Gram-positive bacterias, said fixation step may be achieved by incubating said cells in a 100% ethanol solution.

Following fixation, said cells can be recovered by *e.g.* centrifugation and stored until use at -20°C in a buffered solution at a pH of about 7 (PBS buffer, for example) containing approximately 50% ethanol.

In a preferred arrangement of this embodiment of the invention, said fixation is followed by a dehydration step (or drying step) prior to said contacting phase. Said dehydration step can thus be carried out by placing said sample in contact with at least one ethanol solution, preferably with a

series of ethanol solutions of increasing concentration, for example by placing the sample in a 70%, 80% and then 95% ethanol solution.

Advantageously, said contacting phase is performed by placing the sample in contact with said at least one probe in the presence of a solution hereafter called "hybridization solution", which comprises a denaturing agent such as sodium dodecyl sulfate (SDS) at a concentration in a 0,001-0,1% range, preferably on the order of 0.01%; Tris-HCl , pH of about 8 at a concentration in a 0,001-0,1M range, preferably on the order of 0.02M; and a salt such as sodium chloride at a concentration in a 0,1-1,5M range, preferably on the order of 0.9M. Such a contacting is advantageously performed for an incubation time comprised between about 10 minutes and about 2 hours, and at an hybridization temperature, which is preferably the optimal temperature. For each oligonucleotide probe, the hybridization conditions (temperature; concentration of salts and denaturing agents) can be indeed optimized so as to improve the specificity of the oligonucleotide probe for the corresponding RNA sequences found in the target cells. When a plurality of oligonucleotide probes is used simultaneously, these hybridization conditions can be chosen so as to take into account the optimal conditions peculiar to every probe.

It is very advantageous for the extraction of said at least one probe to be performed following the removal of excess and unbound probe or of non-specifically associated probe material placed in contact, notably by washing with a solution hereafter called "wash solution". Such a "wash solution" advantageously comprises a denaturing agent such as sodium dodecyl sulfate (SDS) at a concentration in a 0,001-0,1% range, preferably on the order of 0.02%; Tris-HCl pH of about 8 at a concentration in a 0,001-0,1M range, preferably on the order of 0.02M, and a salt such as sodium chloride at a concentration in a 0,01-0,9M range, preferably on the order of 0.1M. The formulation of the « wash solution » (*e.g.* salt and denaturant nature and/or concentration) is adjusted so as to achieve the appropriate stringency ; *i.e.* the stringency necessary to the removal of non-specifically

associated probe. Thanks to such a washing step, the extraction step will be performed only on those probes which have become effectively hybridized to the desired target(s).

According to a preferred embodiment of the invention, said extraction is performed by placing the microorganisms potentially present under conditions to denature enabling the denaturation of every probe specifically associated with its target sequence, notably in the presence of a probe-target denaturing agent such as one that will separate duplex DNA/DNA or DNA/RNA, and in particular the probe - target duplex under consideration, and at a temperature higher than the melting temperature of the probe under consideration, notably at a temperature of about 100°C. According to a particularly preferred embodiment of the invention, said denaturing agent is formamide. Said extraction is then performed by incubating said microorganisms in formamide at 100°C for 10 minutes using a controlled temperature incubator. The supernatant may then be recovered for quantification, *e.g.* by centrifugation. To improve detection, said extracted probes can be concentrated notably using a Speed-Vac® prior to measuring the corresponding amount of each probe.

The detection of a target-hybridized probe and the measurement of its amount thus give a qualitative and quantitative analysis of the set of target-microorganisms present in the sample. It is advantageous to perform said detection and amount measurement of the extracted probes by detection and amount measurement of a label associated with or incorporated into each of the contacted probes, such as a radioactive (^{32}P, ^{35}S, ^{125}I), chemiluminescent or fluorescent label. The respective amounts of probes are then measured by quantitation of the corresponding label. It is particularly advantageous to use a fluorescent label, notably fluorescein which can be easily quantified using a fluorescence spectrophotometer.

Different probes, *e.g.* specific probe(s) and/or universal probe(s), can be placed in separate samples, or in the same sample. In the latter case, it is

possible to distinguish each probe used from the others during the detection step, for example by giving to each one its own specific label (*e.g.* different fluorochromes).

The method of the invention can be applied to a variety of samples. Samples for which an analysis using the method of the invention is of particular interest include those taken from fluids such as natural water, industrial water, industrial effluents, municipal wastewater, industrial sludge, thermal mud, food liquid or gel, fermentation medium, air, gas, aerosol; samples from a building ventilation duct, air conditioning duct; samples from edible solid, soil; samples from medical apparatus; human or animal samples such as blood, urine, vaginal or intestinal flora.

The method of the invention utilizes neither microbiological culture, nor microscopy, nor an *in vitro* amplification step (like PCR) and does not require any cell lysis step. It is reproducible, simple, fast (less than 3 hours), low-cost and does not require specially trained personnel. The method of the invention offers the additional advantage of being easy to automate.
The method of the invention notably provides a qualitative and quantitative measurement of the microbiological or sanitary status of said sample and, consequently, of the product from which said sample is taken. The method of the invention can therefore advantageously be combined with an alarm function relating to the quality, safety and/or sanitary monitoring of the product from which the sample is taken, notably as part of an industrial production line.

When the threshold value or set point is exceeded, the method of the invention permits the corresponding quality, safety and/or sanitary alarm to be triggered. It also permits the automatic or feedback control of a microbiological removal or enrichment process.

This invention also relates to the application of said method to *in vitro* diagnostics of infectious diseases.

Beyond applications of the "status or condition measurement" and "alarm" types, this invention relates in particular to the application of said method for the automatic or feedback control of microbiological processes such as methane fermentation of liquid manure, treatment of organic effluents, sewage treatment processes such as activated sludge treatment; or to the automatic or feedback control of a process aimed at removing or preventing the growth of microorganisms.

Thus, the method of the invention may be advantageously applied to the detection of foam formation during the implementation of activated sludge processes and/or to the feedback control of a process aimed at removing or preventing the development of such foams.

Other features and advantages of the invention will further become apparent in the following exemplary embodiments, which are given for illustrative and non-limitating purposes.

EXAMPLE 1: qualitative and quantitative analysis of a sample of sewage treatment reactor effluent

a) Fixation step

Samples of effluent from sewage treatment activated sludge reactors are mixed and then washed three times using a phosphate buffer solution (PBS phosphate-buffered saline) at pH 7. The sample is then incorporated into three volumes of a 4% paraformaldehyde solution and incubated for 3 to 12 hours at 4°C. Following centrifugation the supernatant is removed and the sample is again mixed with a phosphate-buffered saline solution (PBS) at pH 7. An equal volume of ethanol is added and the sample can be stored at -20°C until use.

b) Dehydration step

The fixed sample is centrifuged after adding 1 ml of 70% ethanol over the residue and resuspending the cells. The mixture is centrifuged for 5 minutes then the supernatant is removed. This procedure is repeated with 80% ethanol and then again using 95% ethanol.

c) Hybridization step

A water bath is prepared at the hybridization temperature required by the probe being used (the temperature depends on the length and sequence of the probe). In the example reported here, the following probes were used:

Probe Nb 1000 specific to the *Nitrobacter* genus, with sequence SEQ ID n°1: 5' TGCGACCGGTCATGG 3'

Probe Nso 1225, specific to Ammonia-oxidizing β proteobacteria, with sequence SEQ ID n°2: 5' CGCCATTGTATTACGTGTGA 3'

Probe S Univ-1390, a universal probe for any microorganism, with sequence SEQ ID n°3 : 5' GACGGGCGGTGTGTACAA 3', and

Probe S Bac338, specific for bacteria, with sequence SEQ ID n°4: 5' GCTGCCTCCCGTAGGAGT 3'.

These probes were synthesized, purified by High Performance Liquid Chromatography (HPLC), then fluorescein-labeled at the 5' end. They are available from Operon Technologies of Alameda, California (USA) or, in France, from the Genset company based in Paris (among others).

The cells obtained from the dehydration step are resuspended in 400 µL of a hybridization solution comprising (for 10 mL): NaCl 5M 1.8 mL; Tris-HCl 1M 200 µL; SDS (sodium dodecyl sulfate) 5 µL; distilled excipient water 8 mL, for ten mL. After each probe is labeled by a fluorochrome, the necessary quantity of each probe is added (here, 1.5 nanomoles). The cells in the hybridization solution in contact with the probes are incubated for 10 minutes to 2 hours at the hybridization temperature. The hybridization samples are centrifuged and supernatants are removed.

d) Washing step

Following hybridization, the cells are washed twice for 15 minutes each time at the hybridization temperature, in a buffered washing solution comprising, for 50 mL, NaCl 5M 1 mL; Tris-Hcl 1 M 9 mL; SDS 20% 50 µL. The formulation of the « washing solution » (*e.g.* salt and denaturant) is adjusted according to need of achieving appropriate stringency, *i.e.* removal of non-specifically associated probe.

e) Extraction of the fluorescence by elution

300 µL of formamide heated to 100°C is added to the samples obtained from the washing step, and the residue is gently resuspended. Each tube is placed in 100°C for 10 minutes, preferably using a controlled temperature incubator. Centrifuge for 10 minutes. The supernatant is recovered and stored in the dark until it can be analyzed by fluorescence spectroscopy. The fluorescence is quantified using a spectrofluorometer. The amounts measured in probes Nb1000 and Nso 1225 correspond to the relative amounts of *Nitrobacter* bacteria and Ammonia-oxidizing β proteobacteria contained in the sample. These amounts are compared with those measured for universal probe S Univ-1390 and bacteria probe S Bac 338.

This gives a percentage ratio (the relative proportion) of the microorganisms contained in the sample, which are respectively *Nitrobacter* and Ammonia-oxidizing β proteobacteria.

It is understood that this invention is not limited to the embodiments described and illustrated herein, but covers all variants thereof.

CLAIMS

1. A method of qualitative and quantitative analysis of the microbial population(s) potentially present in a sample, characterized in that it comprises:

- contacting the microorganisms potentially present in said sample with at least one RNA-targeted oligonucleotide probe, hereafter called specific probe, able to target a desired microbiological population, under conditions favourable to *in situ* hybridization in whole cells,
- extracting by separation from their target and elution outside said cells those probes which have become hybridized,
- detecting the extracted probes and measuring the amount thereof or their respective amounts.

2. A method according to Claim 1, further characterized in that said at least one specific probe is chosen among the group consisting of Nb 1000 (SEQ ID N°1) and Nso 1225 (SEQ ID N°2).

3. A method according to Claim 1 or 2, further characterized in that said microorganisms potentially present in said sample are contacted with another probe, hereafter called universal probe, serving to normalize results obtained with probes targeting specific phylogenetic groups of microorganisms.

4. A method according to Claim 3, further characterized in that said universal probe is chosen among the group consisting of S Univ-1390 (SEQ ID N°3) and S Bac 338 (SEQ ID N°4).

5. A method according to any one of the preceding claims, further characterized in that said specific and/or universal probe(s) is a (are) *r*RNA-targeted probe(s).

6. A method according to any one of the preceding Claims, further characterized in that said microorganisms potentially present in said sample are extracted from said sample, notably by centrifugation.

7. A method according to any one of the preceding claims, further characterized in that said contacting is performed following fixation of said cells.

8. A method according to claim 7, further characterized in that said fixation of the cells is achieved by incubation of said cells in a solution of less than 10% paraformaldehyde, and preferably about 4%, for 3 to 12 hours at 4°C.

9. A method according to claim 7 or 8, further characterized in that said fixation is followed by a dehydration step, prior to said contacting step.

10. A method according to claim 9, further characterized in that said dehydration step is performed by placing said sample in contact with at least one ethanol solution, and preferably with a series of ethanol solutions of increasing concentrations.

11. A method according to any one of the preceding claims, further characterized in that said contacting is performed by placing said sample in contact with said at least one probe in the presence of a solution, hereafter called hybridization solution, which notably comprises a denaturing agent such as sodium dodecyl sulfate (SDS) at a concentration in a 0,001-0,1% range, preferably on the order of 0.01%, Tris-HCl, pH of about 8 at a concentration in a 0,001-0,1 M range, preferably on the order of 0.02M; and a salt such as sodium choride at a concentration in a 0,1-1,5 M range, preferably on the order of 0.9 M .

12. A method according to any one of the preceding Claims, further characterized in that said contacting phase is performed for an incubation time of about 10 minutes to about 2 hours, and at the optimal hybridization temperature.

13. A method according to any one of the preceding claims, further characterized in that said extraction of said at least one probe is performed following removal of the excess and unbound probe or of non-specifically associated probe material placed in contact, notably by washing with a solution, hereafter called wash solution, which notably comprises a denaturing agent such as sodium dodecyl sulfate (SDS) and a salt such as sodium chloride at concentrations appropriate for achieving the stringency necessary to the removal of non-specifically associated probe.

14. A method according to any one of the preceding claims, further characterized in that said extraction is performed by placing said microorganisms potentially present under conditions enabling the denaturation of every all probe specifically associated with its target sequence, notably in the presence of an agent able to denature the probe-target duplex, and at a temperature higher than the melting temperature of the probe under consideration, notably at a temperature of approximately 100°C.

15. A method according to claim 14, further characterized in that the denaturing agent is formamide.

16. A method according to any one of the preceding claims, further characterized in that said extracted probes are concentrated, notably using

the Speed-Vac®, prior to the measurement of the amount thereof or of their respective amounts.

17. A method according to any one of the preceding claims, further characterized in that said detection and amount measurement of extracted probes is performed by detection and amount measurement of a label associated or incorporated into each of the contacted probes, such as a radioactive, chemiluminescent or fluorescent label, notably such as fluorescein.

18. A method according to any one of the preceding claims, further characterized in that said sample is taken from fluids such as natural water, industrial water, industrial effluent, municipal wastewater, industrial sludge, thermal mud, food liquid or gel, fermentation media, air, gas, aerosol, or is a sample taken from a building ventilation duct, air conditioning duct, sample of food solid, sample of soil, sample from medical apparatus, or is a human or animal sample, such as blood, urine, vaginal or intestinal flora.

19. A method according to any of the preceding claims, further characterized in that it is used in combination with a process for triggering an alarm in connection with quality, safety and/or sanitary monitoring of the product from which said sample has been obtained.

20. A method according to any one of the preceding claims, further characterized in that it is used in *in vitro* diagnosis of an infectious disease.

21. A method according to any one of the preceding claims, further characterized in that it is used in the automatic or feedback control of a microbiological process such as methane fermentation of liquid manure,

treatment of organic effluents, sewage treatment process such as treatment by activated sludge.

22. A method according to any of the preceding claims, further characterized in that it is used in the automatic or feedback control of a process relating to the removal or prevention of the development of microorganisms.

23. A method according to any of the preceding claims, characterized in that it is applied in the detection of foam formation during the implementation of activated sludge processes and/or for the feedback control of a method relating to the removal or prevention of the said foams.

LIST OF SEQUENCES

(1) General information:

(i) Applicant:
(a) Name: Suez Lyonnaise des Eaux
(b) Address: 72 avenue de la Liberté
(c) City: Nanterre Cedex
(e) Country: France
(f) Postal code: 92753

(ii) Title of the invention: Means for qualitative and quantitative analysis of the microbial populations potentially present in a sample

(iii) Number of sequences: 4

(iv) Form readable by computer:

(a) type of storage medium: floppy disk
(b) computer: IBM PC compatible
(c) operating system: PC-DOS/MS-DOS
(d) software: PatentIn Release #1.0, Version #1.30 (OEB)

(2) Information for SEQ ID n° 1:

(i) Characteristics of the sequence:
(a) Length: 15 base pairs
(b) Type: nucleotide
(c) Number of strands: single
(d) Configuration: linear

(ii) Type of molecule: other nucleic acid
(iii) Hypothetical: yes
(iv) Antisense: no
(vii) Immediate source:
(B) Clone: Nb1000

(xi) Description of the sequence: SEQ ID n° 1:
TGCGACCGGT CATGG

(3) Information for SEQ ID n° 2:

(i) Characteristics of the sequence:
(a) Length: 20 base pairs
(b) Type: nucleotide

(c) Number of strands: single
(d) Configuration: linear

(ii) Type of molecule: other nucleic acid
(iii) Hypothetical: yes
(iv) Antisense: no
(vii) Immediate source:
(B) Clone: Nb1225

(xi) Description of the sequence: SEQ ID n° 2:
5' CGCCATTGTA TTACGTGTGA 3'

(4) Information for SEQ ID n° 3:

(i) Characteristics of the sequence:
(a) Length: 18 base pairs
(b) Type: nucleotide
(c) Number of strands: single
(d) Configuration: linear

(ii) Type of molecule: other nucleic acid
(iii) Hypothetical: yes
(iv) Antisense: no
(vii) Immediate source:
(B) Clone: S Univ-1390

(xi) Description of the sequence: SEQ ID n° 3:
5' GACGGGCGGTGTGTACAA 3'

(5) Information for SEQ ID n° 4:

(i) Characteristics of the sequence:
(a) Length: 18 base pairs
(b) Type: nucleotide
(c) Number of strands: single
(d) Configuration: linear

(ii) Type of molecule: other nucleic acid
(iii) Hypothetical: yes
(iv) Antisense: no
(vii) Immediate source:
(B) Clone: S Bac338

(xi) Description of the sequence: SEQ ID n° 4:
5' GCTGCCTCCCGTAGGAGT 3'

DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: Means for qualitative and quantitative analysis microbial populations potentially present in a sample.
the specification of which: (check one)

[ ] is attached hereto. [ ] was filed on Oct. 2 1998 as United States Patent Application Serial No. or PCT International Application Number PCT/EP 9806286, and was amended on _____ 19__ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 CFR § 1.56(a).

Prior Foreign Application(s): I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate listed below, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

(Application No.)	(Country)	(Day/Month/Year Filed)	Priority Claimed
97 12552	FRANCE	October 8, 1997	[X] Yes [ ] No
			[ ] Yes [ ] No
			[ ] Yes [ ] No

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

Application No.	Filing Date

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by 35 U.S.C. § 112, first paragraph, I acknowledge the duty to disclose material information as defined in 37 CFR § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(U.S. Application Serial No.)	(U.S. Filing Date)	(Status--patented, pending, abandoned)

I hereby appoint Elliott I. Pollock, Registration No. 16,906; George Vande Sande, Registration No. 17,276; Burton A. Amernick, Registration No. 24,852; Stanley B. Green, Registration No. 24,351; Richard Wiener, Registration No. 18,741; Townsend M. Belser, Jr., Registration No. 22,956; Morris Liss, Registration No. 24,510; Martin Abramson, Registration No. 25,787; George R. Pettit, Registration No. 27,369; Elzbieta Chlopecka, Registration No. 32,767; Eric J. Franklin, Registration No. 37,134; and Jeffri A. Kaminski, Reg. No. 42,709, my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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[X] *See next page for additional inventors*

DECLARATION FOR PATENT APPLICATION

Page Two

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6-00 Full name of sixth joint inventor (if any): Jodi FLAX
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7-00 Full name of seventh joint inventor (if any): Michaël WAGNER
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Full name of eighth joint inventor (if any): _____
Inventor's Signature _____ Date _____
Residence Address _____
Citizenship _____
Post Office Address _____